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Phytic acid as an efficient low-molecular-mass displacer for anion-exchange displacement chromatography of proteins

Qilie Luo[a], Joseph D. Andrade[b,*]

[a]*University of Utah, Department of Materials Science and Engineering, 122 S Central Campus DR RM 304, Salt Lake City, UT 84112-0560, USA*

[b]*University of Utah, Department of Bioengineering, 50 S Campus Center DR RM 2480, Salt Lake City, UT 84112-9202, USA*

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Abstract

Phytic acid, inositol-hexaphosphoric acid, molecular mass 650, a low-molecular-mass compound, has been identified as a nearly ideal displacer in anion-exchange displacement chromatography for the concentration and purification of model protein mixture. The concentration of low-molecular-mass displacer is a very important parameter for successful separation by displacement chromatography. Displacer concentration influences the formation of the isotachic train and the yield and recovery of the displacement chromatographic process. There is an optimum displacer concentration in which the yield and recovery are highest.

Keywords: Displacers; Phytic acid; proteins

1. Introduction

Protein chromatography continues to play a significant role in biotechnology, serving as an effective analytical tool and a powerful method for large-scale separation [1,2]. Displacement chromatography has become popular in recent decade even though its potential was already recognized by Tiselius in the early 1940s [3–9]. This progress has been driven by the development of biotechnology and the specific advantages of the method [10,11], including:

1. the feed concentration generally does not affect process efficiency;
2. products can be concentrated in the column effluent;
3. the concentration of displacer can easily adjust the speed and efficiency of the separation; and
4. peak tailing is greatly reduced.

Kundu and Cramer [12] had pointed out that, even though a great many systems have been tried, the lack of effective and nontoxic displacers hampers the wide application of displacement chromatography in biotechnology. Generally, whether displacement chromatography is successful or not depends greatly on the efficiency of displacer. The displacer, with a higher surface affinity for the adsorbent than any of the feed components, effectively competes with the

*Corresponding author. Tel.: +1-801-581-4378; fax: +1-801-585-5361.

E-mail address: Joe.Andrade@m.cc.utah.edu (J.D. Andrade)

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feed components for adsorption sites on the stationary phase under nonlinear conditions [20]. Displacers can be low-molecular-mass compounds or macromolecules. Macromolecular displacers can be synthetic polyelectrolytes or proteins. The displacer should meet a number of requirements: nontoxic, stable, detectable, soluble, inert (no interaction with other feed components), high affinity, and be highly uniform, cheap, and reusable [13]. Generally, it is not easy for a displacer to rigorously satisfy all these requirements.

An important recent advance in displacement chromatography was the discovery that low-molecular-mass compounds can be successfully used as displacers for protein purification [16]. Compared with macromolecular displacers, low-molecular-mass displacers have significant operational advantages:

1. they can be easily separated from the purified protein components;
2. the economics of the process can be improved because of the relatively low prices of low-molecular-mass displacers; and
3. column regeneration is facilitated.

Phytic acid (Fig. 1) is a nearly ideal displacer. When the pH>7.2, there are at least eight negative charges on a small inositol molecule. Phytic acid has a strong affinity for anion-exchangers under appropriate conditions. Phytic acid is abundant plant constituent in cereals and can be easily separated from this food source. Phytic acid meets most of the requirements suggested by Kasper et al. [13] for an ideal displacer. We have evaluated phytic acid as displacer.

In this paper, a strong anion-exchanger was used as adsorbent. A mixture of β-lactoglobulins A and B is employed as model proteins in order to evaluate the separation resolution using phytic acid as a low-molecular-mass displacer. The two forms of β-lactoglobulins A and B differ in p*I* by approximately 0.1 unit. Aspartic acid (64) and valine (118) in A is replaced by glycine and alanine, respectively, in B. The program ''p*I* protein 1.0v1'' was used to draw titration curves, thus determining the suitable pH. Frontal analysis was used for measuring adsorption isotherms, and then determining the feed concentration. Finally, the column chromatographic separation was carried out with the optimum conditions determined by frontal analysis and titration curves.

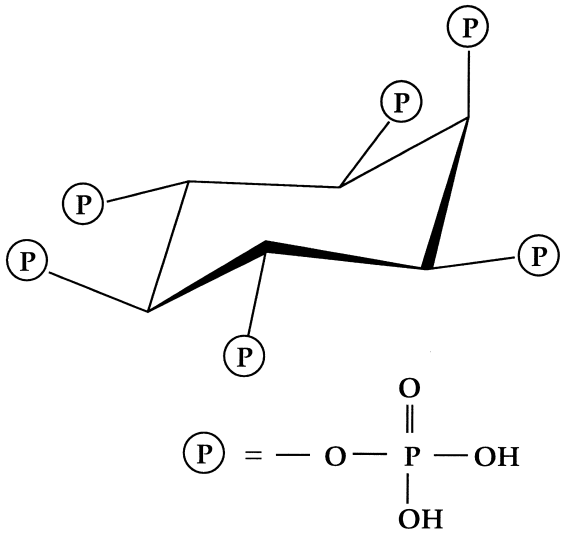

Fig. 1. The chemical structure and titration properties of phytic acid [25].

2. Experimental

2.1. Materials

Two adsorbents, strongly basic, quaternary amine (QA-52) bearing and diethylaminoethyl-derivatized (DE-52) microgranular and preswollen type celluloses (strong and weak anion-exchangers, respectively), were manufactured by Whatman (Clifton, NJ, USA). QA-52 medium is fully ionized and bears constant charges in the pH range 2–12; but DE-52 medium depends on pH. Phytic dodecasodium salt (PN12), phytic dipotassium salt (PK2), β-lactoglobulin A, β-lactoglobulin B, and a mixture β-lactoglobulins A and B, were all purchased from Sigma (St. Louis, MO, USA). Buffer, N,N-bis-(2-hydroxyethyl)-2-aminoethanesulphonic acid (free acid and Ultra Grade), N,N′-bis(2-hydroxyethyl)-2-amino-

ethanesulfonic acid, was obtained from Calbiochem-Novabiochem (La Jolla, CA, USA). All other chemicals used were of analytical grade.

2.2. Equipment

PCI photon counting spectrofluorometer was manufactured by ISS (Champaign, IL, USA). Fast protein liquid chromatography systems including a fraction collector and columns, HR 100×10 mm I.D., HR 100×5 mm I.D., and HR 50×5 mm I.D., respectively, were obtained from Amersham Pharmacia (Piscataway, NJ, USA).

2.3. Solutions

Solutions used for equilibration of ion-exchanger, package of columns, frontal analysis, thin layer ion-exchange chromatography, column ion-exchange chromatography, and displacement chromatographic developments contained sodium phosphate, phytate, BES, and sodium chloride (Table 1).

2.4. Titration curves

Generally, there are three methods, including chemical titration, calculation, and isoelectric focusing, to get titration properties of proteins. If the amino acid composition of the protein is known, the calculation method is preferred; if not, the chemical titration method is more suitable. However, for complex samples, the isoelectric focusing method is better. In this paper, the calculation method was used to achieve titration curves.

Table 1
The compositions and properties of buffer solutions[a]

Name	$NaH_2PO_4 \cdot H_2O$ (*M*)	$Na_2HPO_4 \cdot 7H_2O$ (*M*)	BES (*M*)	NaCl (*M*)
NPB1	0.0032	0.0168		
NPB2	0.0032	0.0168		0.2
NPB3	0.0032	0.0168		0.1
BES1			0.02	
BES2			0.02	0.2
BES3			0.02	0.1
BES4			0.02	0.25
CAB			0.025	0.025
REB[b]			0.025	0.25
CPB[c]			0.025	0.1

[a] pH for all buffer solutions is 7.50.
[b] Regenerant buffer.
[c] Corrected purpose buffer.

2.5. Frontal analysis

A column (HR 50×5 mm I.D.) packed with 0.04 ml QA-52 medium was used for frontal analysis, which was modified from Ref. [21]. The final bed height was 2 mm. First, the column was equilibrated with carrier buffer (Table 1), then 2, 4, 8, 16, 32 ml protein solutions of corresponding different concentrations, 0.64, 0.32, 0.16, 0.08, 0.04 m*M*, respectively, were flown through the column under the gravity force, and the various whole effluents were collected. Finally, the protein concentrations were measured by intrinsic UV fluorescence. The samples of diluted effluent were analyzed using a PCI photon counting spectrofluorometer (ISS) at $\lambda_{ex.}=280$ nm and $\lambda_{em.}=340$ nm. The maximum protein concentration was limited to less than 20 μm/ml to maintain a pseudo-linear state between protein concentration and the fluorescence intensity. Temperature was controlled at 21±1°C. The subtract between the protein concentrations of original solution and effluent, respectively, multiplied by the effluent volume was the adsorbed protein amount.

2.6. Effluent analysis

Displacement chromatography requires the use of an on-line analyzer because the detectors usually used do not provide sufficient information on the boundary regions. In this work, two separation techniques were used: ion exchange thin layer chromatography (EXTLC) and ion-exchange column chromatography (EXCC).

2.6.1. EXTLC

EXTLC, due to its simplicity, convenience, and speed, was employed to quickly screen the fractions collected from displacement chromatography. The operation processes of EXTLC are in Ref. [14]. Here are given some important development parameters, including DE-52 as adsorbent; sodium (Na) phosphate buffer as equilibrated solution; and the solution made up of 20% NPB2 and 80% NPB3 as eluent,

which eluted β-lactoglobulin B but not β-lactoglobulin A.

2.6.2. EXCC

EXCC was used for quantitatively measuring β-lactoglobulins A and B in the fractions screened by EXTLC. A HR 100×10 mm I.D. column packed with QA-52 was used. The final bed height is 88 mm. The chromatography was carried through FPLC systems (Amersham Pharmacia). Equilibrated solution was BES3. Step gradient elution was used. The solution in the first step is made up of 87% BES2 and 13% BES4 and the solution in the second step made up of 19% BES2 and 82% BES4. The concentrations of β-lactoglobulins A and B in effluent fractions were measured by intrinsic UV fluorescence.

2.7. Displacement chromatography operation

Displacement chromatography was carried out through FPLC systems. The volume of sample loop is 1.5 ml. 1.85 ml of QA-52 medium were packed in a HR 100×5 mm I.D. column at a flow-rate of 1 ml/min. The final bed height was 94 mm. The column was first equilibrated with CAB solution (carrier). The solution of β-lactoglobulins A and B was loaded in the sample loop and pushed into the column by displacer solution (phytic acid in carrier). Effluent fraction, its sizes 0.5 ml or 0.25 ml, respectively, depending on displacer concentrations, was collected through a fractional collector.

3. Results and discussion

3.1. Titration properties of β-lactoglobulins A and B

The titration curves are generally used for determining pH suitable for eluent, or displacer solution. Fig. 2 shows the titration curves of β-lactoglobulins A and B drawn by the program ''p*I* protein 1.0v1'' (Internet: iho@biobase.aau.dk). Fig. 2 shows that the difference in net charge numbers is small in a broad pH range. So, the selection of pH 7.50 is mainly based on the consideration of protein stability and protein binding capacity of adsorbent. β-Lactoglobulins A and B tends to form dimers in pH range 1.8–5.4 [22]. As the pH increases above 5.4, an ionization-linked transition is observed in all variants. The conformation changes and increasing dissociation near pH 7.5 are established immediately on mixing [23]. Groves et al. detected the presence of both reversible and irreversible changes above pH 8.0 [24].

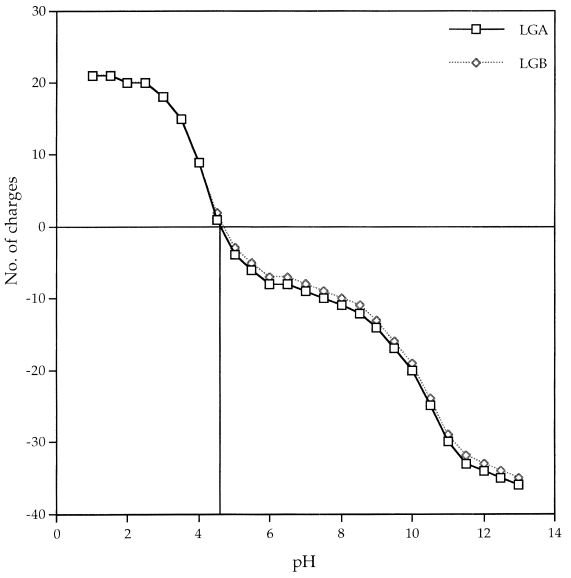

Fig. 2. The relation between the net calculated charges and pH of β-lactoglobulins A and B.

3.2. Adsorption isotherms

The adsorption isotherms are utilized to determine displacer concentrations suitable for displacement separation development. However, we did not measure the adsorption isotherm of phytic acid displacer. For a small molecular mass displacer, such as phytic acid, it is hard to find appropriate units (mole, weight, or equivalent scales) to express displacer concentrations. According to the research of Jen and Pinto [15], the adsorption isotherms will be completely different if the concentration units to express adsorption isotherms are different even for macromolecular displacers. Here we only measured the adsorption isotherms of model protein, β-lactoglobulins A and B. The effects of displacer concentration on the displacement chromatographic separation

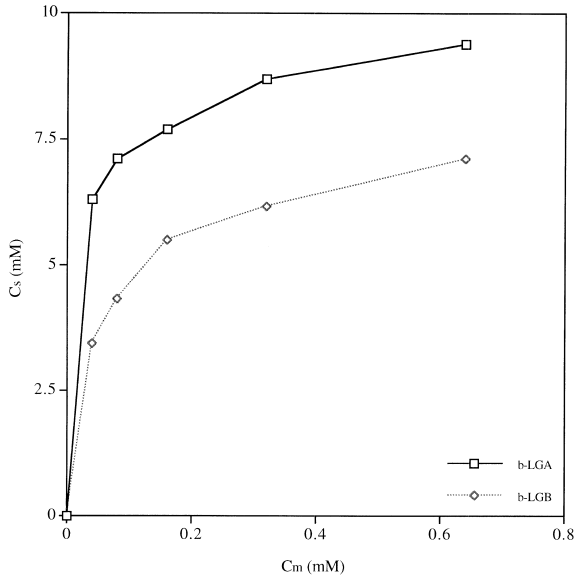

Fig. 3. Adsorption isotherms of β-lactoglobulins A and B at 25 m*M* BES, 25 m*M* NaCl, and pH 7.5 measured by frontal analysis.

were tested directly in the development processes. Fig. 3 demonstrates the adsorption isotherms of β-lactoglobulins A and B. Although there are only two amino acids different between β-lactoglobulins A and B, there is a relatively large difference in adsorption isotherms, which means these two proteins can be separated each other from the view of adsorption isotherms. Moreover, Fig. 3 also shows that the concentrations of β-lactoglobulins A and B can be selected from a relatively broad range.

3.3. The development of displacement chromatography

3.3.1. The effect of displacer concentrations

Displacement chromatograms of β-lactoglobulins A and B in Fig. 4 show some interesting phenomena. First, when displacer (phytic acid) concentration increased from 10 m*M* to 40 m*M* in carrier (CAB), the isotachic trains are gradually formed. The formation of an isotachic train is very important, as this gives the highest recovery yield, although it does not permit the highest possible production rate [17]. Second, when the displacer concentration is increased, the elution properties are changed from overload isocratic elution in Fig. 4a to displacement

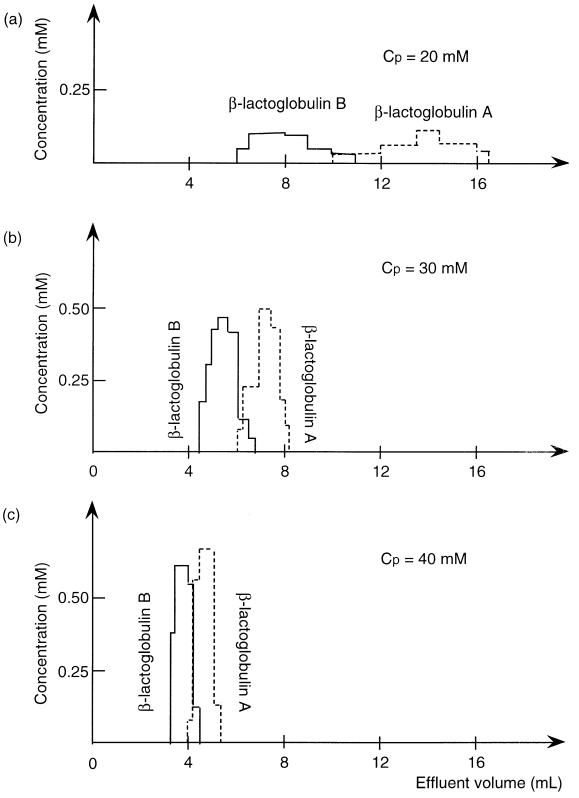

Fig. 4. Displacement chromatograms of β-lactoglobulins A and B. Column, QA-52, HR 100×5 mm I.D.; Carrier, 25 m*M* BES, 25 m*M* NaCl, and pH 7.5 with 0.5 ml/min flow-rate; Feed, 1.5 ml of a mixture of β-lactoglobulins A and B at each concentration of 0.50 m*M* in the carrier with 0.1 ml/min flow-rate; Displacer, phytate composed of PN12 and PK2 in carrier, (a) 20 m*M*, (b) 30 m*M*, and (c) 40 m*M* phytate, respectively; Fraction size, (a) 500 μl, (b) and (c) 250 μl; Regenerant, 25 m*M* BES, 1.0 *M* NaCl, and pH 2.5 with flow-rate 0.5 ml/min.

development in Fig. 4c. This phenomenon is consistent with that reported by Liao et al. [5] although they used macromolecular compound as displacer. It should be pointed out that the characteristic of displacement chromatography is that there is an isotachic train or there is a plateau concentration in displacement chromatogram. In displacement chromatography, it often occurs that the concentration of some of the feed components in the isotachic train is higher than their concentration in the feed. This is in contrast with overload elution chromatography, where dilution of the feed always occurs [19]. From this viewpoint, overload elution chromatography is

shown in Fig. 4a because of the dilution of the feed components; displacement chromatography is shown in Fig. 4(b and c), due to the concentration increase in the feed components.

The results for the 10 m*M* displacer concentration are not shown in Fig. 4 because at this displacer concentration, β-lactoglobulins A and B cannot be eluted or stay in the column. When carrier pH is 7.5, phytic acid has about eight net charges (Fig. 1). Ionic strength is calculated according to the equation,

$$I = \frac{1}{2}\sum_i c_i z_i^2 \quad (1)$$

where I is ionic strength, c_i is ion concentration, and z_i is the charge number of the ions. For phytic acid, when $c = 10$ m*M* and $z = 8$, $I = 0.32$ *M*. From the step-gradient elution data [18], if NaCl is used as elution salt, the needed ionic strength is only 0.23 *M* even for β-lactoglobulin A, which means that phytate cannot be simply considered as the common salt.

Generally, β-lactoglobulins A and B cannot be separated by isocratic elution if a common salt like NaCl is used. However, when a low concentration of phytic acid was used in displacement chromatography, β-lactoglobulins A and B can be separated under overload elution. In displacement chromatography, the mobile phase flow-rate is much lower than that in isocratic elution chromatography, which itself seems to contribute to separation resolution because the mass transfer resistance is greatly reduced through the decrease in mobile phase flow-rate. Cysewski et al. [26] modeled multivalent ion-exchange for mass overload conditions. Their simulation results predicted that peak tailing due to column overloading may be reduced using a counter ion of higher valence; they thought that this conclusion may be useful for optimizing separations in preparative chromatography. Phytate is a counter ion with high valence (at least eight), so phytic acid also made contributions to separation resolution in isocratic overload elution chromatography of proteins. Further research is needed in this respect.

3.3.2. Yield and recovery

Table 2 shows yields and recoveries of the process. The yields and recoveries depend on the displacer concentrations. There is an optimum displacer concentration (30 m*M*), in which the yield and recovery are highest. When phytic acid concentration is less than 30 m*M*, it seems that there is protein left in the column perhaps due to the formation of dimer. There is a stronger interaction between protein dimer and adsorbent because the interaction sites in dimers are almost two-times that of monomers. β-Lactoglobulins prefer to form dimers when ionic strength is high, protein concentration is high, and pH is equal or less than 7.5 [27]. When phytic acid concentration is 40 m*M*, the yield and recovery are also low. As the displacer concentration increases, the hydrophobic interaction between protein and adsorbent also increases. The low yield and recovery may result from protein being retained in the column due to hydrophobic interaction, increase in higher displacer concentrations, and relatively higher hydrophobicity of protein β-lactoglobulins [28].

Table 2
Yields and recoveries of β-lactoglobulins A and B purified by displacement with a purity of 100%

Displacement conditions	Yield (mg)	Recovery (%)
β-Lactoglobulin A		
as in Fig. 4a	6.64	53.6
as in Fig. 4b	10.8	87.1
as in Fig. 4c	6.80	55.0
β-Lactoglobulin B		
as in Fig. 4a	6.11	49.4
as in Fig. 4b	8.23	66.5
as in Fig. 4c	7.39	59.7

4. Conclusions

Phytic acid has been evaluated as an efficient displacer for anion-exchange displacement chromatography. Phytic acid as a small molecular mass displacer has its own intrinsic elution property and displacement properties. When the phytic acid concentration in the carrier is increased, the elution properties are changed from isocratic elution to displacement separation. The yields and recoveries of the displacement chromatographic process also depend on the displacer concentrations. There is an optimum displacer concentration, at which the yield

and recovery are highest and an isotachic train is formed. The concentration of low-molecular-mass displacer in displacement chromatography is the most important parameter.

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